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Ecdysone induces transcription and amplification in *Sciara coprophila* DNA puff II/9A

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Abstract

DNA replication is normally tightly regulated to ensure the production of only one copy of the genome per cell cycle. However, DNA puffs of the salivary gland giant polytene chromosomes of *Sciara coprophila* undergo DNA amplification during the normal course of development, overriding this control. This developmental strategy provides more template for the production of large amounts of protein needed for pupation. We have focused on DNA puff II/9A, which amplifies ~17-fold over the rest of the genome. Evidence presented here suggests that DNA amplification at this locus is controlled by the steroid hormone ecdysone, the master regulator of insect development. Explanted, pre-amplification stage salivary glands undergo premature amplification when incubated with ecdysone. Injection of ecdysone into pre-amplification stage larvae induces amplification. Ecdysone also induces transcription of the II/9A genes. We report the presence of a putative ecdysone response element directly adjacent to the origin recognition complex (ORC)-binding site in the II/9A origin and demonstrate that it is efficiently bound by the *Sciara* ecdysone receptor. These results implicate ecdysone in the regulation of DNA amplification in *Sciara* and suggest the ecdysone receptor may be the elusive amplification factor. This would be a new role for this transcription factor.

Keywords: DNA amplification; Ecdysone; *Sciara coprophila*; DNA puff; EcRE; Amplification factor

Introduction

The initiation of DNA replication is a paramount control point in the regulation of the cell cycle, because once replication begins the cell is destined to complete progression through the cell cycle. Replication is tightly controlled so that each origin fires once and only once ensuring the production of one copy of the genome per round of the cell cycle. What cellular mechanisms ensure that the "The Rule of DNA Constancy" (Boivin et al., 1948; Mirsky and Ris, 1951; Swift, 1950) will be obeyed? One way to approach this question is to study instances when this regulation is subverted and DNA amplification occurs. Perhaps the most famous example is methotrexate resistance in mammalian cells arising from the amplification of the dihydrofolate reductase (DHFR) locus (Alt et al., 1978; reviewed in Hamlin, 1992). In addition, the ribosomal RNA genes become amplified in amphibian oocytes (Brown and Dawid, 1968; Gall, 1968), in the transcriptionally active macronuleus in *Tetrahymena thermophila* (Yao et al., 1974), and in Pterygotan (winged) insect oocytes and nurse cells (Gall et al., 1969; Kubrakiewicz and Bilinski, 1995; Troster et al., 1990). However, these are extrachromosomal events, as the amplified locus is excised from the DNA and replicates as an episome (amphibian oocytes and Pterygotan insects) or mini-chromosome (*Tetrahymena*).

In dipteran insects, two developmentally regulated examples of intrachromosomal amplification have been described (reviewed in Claycomb and Orr-Weaver, 2005). We study DNA amplification found in the giant polytene chromosomes in the larval salivary glands of sciarid flies. The entire genome in each nucleus of the gland is endoreduplicated (in *Sciara* to 4096C in males and 8192C in females) without intervening mitoses (Rasch, 1970b). The daughter chromatids of both homologous chromosomes remain synapsed together in tight register forming giant polytene chromosomes. Superimposed

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on the last endocycle several origins of replication fire repeatedly, resulting in localized areas of DNA amplification. Bidirectional replication from these origins (Liang et al., 1993) is consistent with an "onion-skin" model of nested replication forks (Osheim et al., 1988; Spradling, 1981). Subsequent transcription at these loci causes the polytene chromosomes to become distended, resulting in gigantic "DNA puffs" (Gabrusewycz-Garcia, 1964; Poulson and Metz, 1938; Rudkin and Corlette, 1957), so-called because they contain amplified DNA (Crouse and Keyl, 1968; Glover et al., 1982; Rasch, 1970a; Wu et al., 1993). This is in contrast to *Drosophila* salivary glands which only have "RNA puffs", sites of intense transcription but no DNA amplification (Rasch, 1970b; Rudkin et al., 1955). In fact, both types of puff are found in sciarid salivary glands.

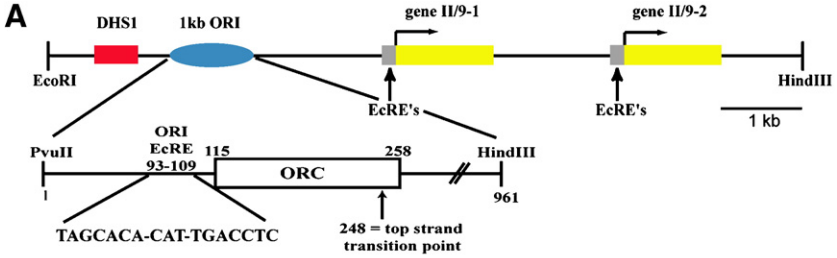
The other example of intrachromosomal DNA amplification occurs during oogenesis in *Drosophila* follicle cells where the two major and two minor chorion loci become amplified (Claycomb et al., 2004; Spradling et al., 1980; Spradling, 1981). These loci encode proteins involved in making the chorion (eggshell). The major chorion locus on chromosome 3 amplifies 60- to 80-fold (Claycomb et al., 2004; Delidakis and Kafatos, 1989; Spradling, 1981). Deletion analysis identified the amplification regulator ACE3 (Amplification Control Element for the third chromosome) (Carminati et al., 1992; Delidakis and Kafatos, 1989; Orr-Weaver and Spradling, 1986) and two-dimensional gel analysis mapped the origin ~1.5 kb downstream in a region called oriβ (Heck and Spradling, 1990). These two *cis*-elements are sufficient to direct DNA amplification (Lu et al., 2001). Amplification of the chorion loci also follows a few endoreduplications (only to 16C). The endocycles are regulated by the normal cell cycle machinery with cyclin E levels rising and falling during each endocycle (Calvi et al., 1998; Follette et al., 1998; Weiss et al., 1998). Cyclin E remains high at the end of the last endocycle, halting genome wide replication. Regardless, the chorion loci origins remain able to fire (Calvi et al., 1998). Hence, DNA amplification presents a unique opportunity to study a specific origin that circumvents the normal regulation of rereplication.

Mutational analysis has shown that the proteins involved in normal DNA replication are also used during amplification, making developmentally regulated DNA amplification an attractive model to study the regulation of DNA replication (Claycomb and Orr-Weaver, 2005). Mutations which disrupt amplification result in a thin eggshell phenotype and female sterility (Snyder et al., 1968). These include pre-replication complex (pre-RC) components like the origin recognition complex (ORC) (Landis et al., 1997), double-parked (dup/cdt1) (Whittaker et al., 2000) and the MCM complex (Schwed et al., 2002). Mutations in proteins involved in initiation and fork progression like chiffon (dbf4-like) (Landis and Tower, 1999) and PCNA (Henderson et al., 2000) reduce amplification. Additionally, two genes necessary for replication, mus101 (Yamamoto et al., 2000) and humpty-dumpty (Bandura et al., 2005), have recently been implicated in amplification. Moreover, chromatin immunoprecipitation and in vitro binding assays demonstrated that ORC binds ACE3 and oriβ directly (Austin et al., 1999; Chesnokov et al., 1999).

We have studied the largest and earliest appearing DNA puff, on chromosome II at position 9A (II/9A), in late fourth instar larvae of the fungus fly, *Sciara coprophila* (Gabrusewycz-Garcia, 1964; Rasch, 1970a). Two- and three-dimensional mapping studies determined that the majority of replication initiation events are confined to a ~1-kb fragment (ORI = origin) during amplification (Fig. 1A) (Liang et al., 1993; Liang and Gerbi, 1994). Interestingly, nascent strand analysis revealed a larger initiation zone of 8–9 kb during regular mitotic cell cycles and endocycles, which becomes focused to the 1-kb ORI upon the start of amplification (Lunyak et al., 2002). A binding site for ORC has been mapped in the 5′ portion of the 1-kb ORI (Bielinsky et al., 2001). The start site for leading strand synthesis on the top strand, identified by replication initiation point (RIP) mapping, is located near the 3′ end of the ORC-binding site (Bielinsky et al., 2001). Additionally, we have discovered a DNase I hypersensitive site (DHS1) (Urnov et al., 2002) about 600 bp upstream of the 1-kb ORI, which appears to form the upstream boundary for initiation of replication in all developmental stages (Lunyak et al., 2002). The II/9A locus contains two genes (II/9-1 and II/9-2) which are 85% identical and encode secreted proteins with α-helical coiled coil domains and are likely components of the of the pupal case (DiBartolomeis and Gerbi, 1989).

The II/9A locus becomes amplified about 17-fold (Wu et al., 1993) resulting in about 140,000 copies of the locus per nucleus in females. After amplification is completed, a burst of transcription from the locus ensues (Wu et al., 1993). Therefore, site-specific DNA amplification is likely an adaptation which provides substantial template for the production of mRNAs that encode proteins needed in vast quantity and in a short period of time for the subsequent developmental stage. The flies avoid any deleterious effects of having "extra" DNA because the salivary glands undergo histolysis during pupation.

The developmental cues which direct amplification and the underlying mechanisms which restrict amplification to specific chromosomal sites remain unclear. Several lines of evidence suggest that the steroid hormone, ecdysone, the master regulator of insect development, directs amplification in *Sciara*. In both *Drosophila* (Thummel, 1996) and *Rhynchosciara* (Stocker et al., 1984), a strong burst of ecdysone is produced at the end of the last larval instar, inducing genes (Beckstead et al., 2005) which direct the transition to pupation. The ecdysone receptor is an obligate heterodimer of the ecdysone receptor (EcR) (Koelle et al., 1991) and ultraspiracle (USP) (Thomas et al., 1993; Yao et al., 1992, 1993). Ecdysone induces RNA puffs in *Drosophila* salivary glands (Ashburner et al., 1974). Similarly, DNA puffs can be induced prematurely by injection of ecdysone in sciarid flies (Amabis and Amabis, 1984a; Crouse, 1968; Stocker and Pavan, 1974). Moreover, a ligature blocking the flow of ecdysone from the prothoracic gland associated with the brain to the rest of the body prevents amplification (Amabis et al., 1977; Amabis and Amabis, 1984b). We have demonstrated that the promoter for gene II/9-1 is regulated by ecdysone in a tissue and temporal specific manner in transgenic *Drosophila* (Bienz-Tadmor et al., 1991). In contrast, neither constructs containing a part of the II/9A locus (Bienz-Tadmor et al., 1991) nor the



B

	Left half site								Right half site						
	-7	-6	-5	-4	-3	-2	-1	0	+1	+2	+3	+4	+5	+6	+7
Consensus	**R/c**	**R**	**G**	**G/t**	**T**	**C/t**	**A**	**A/t**	**T**	**G**	**A**	**C/a**	**C/a**	**Y**	**Y**
PAL1	G	A	G	G	T	C	A	A	T	G	A	C	C	T	C
Hsp27	G	G	G	T	T	C	A	A	T	G	c	A	C	T	T
II/9-1 EcRE 1	t	A	t	T	T	C	c	gt	T	G	A	A	C	a	a
EcRE 2	G	t	G	G	a	C	A	tat	T	G	A	C	C	a	a
EcRE 3	A	c	G	T	a	C	g	aac	T	G	A	C	C	T	T
II/9-2 EcRE 1		G	G	T	T	C	A	ag	T	T	A	g	g	g	
EcRE 2		A	G	T	T	C	A	ca	T	c	g	C	A	T	
II/9A ORI	t	A	G	c	a	C	A	cat	T	G	A	C	C	T	C

R= G or A; Y= C or T

Fig. 1. Introduction to *Sciara* DNA puff II/9A. (A) A map of the major landmarks in the II/9A locus showing the relative location of the DNase hypersensitive site DHS1, 1-kb ORI and genes II/9-1 and II/9-2. A magnified view of the 5′ end of the 1-kb ORI is shown below indicating the location of the ORC-binding site, top strand DNA replication start site and the ORI EcRE. (B) A comparison of EcREs from *Drosophila* and *Sciara* including: PAL1, the "perfect" *Drosophila* EcRE elucidated by in vitro evolution experiments (Vögtli et al., 1998) and the hsp27 EcRE (Riddihough and Pelham, 1987) from *Drosophila*. The consensus sequence is compiled from analysis of *Drosophila* sequences (Riddiford et al., 2000). Below the double line are the EcREs we identified in the promoters of genes II/9-1 and II/9-2 and the *Sciara* II/9A 1-kb ORI.

Rhynchosciara DNA puff C3 (Soares et al., 2003) were able to amplify in transgenic *Drosophila* suggesting that a factor unique to the salivary glands of sciarid flies is necessary.

In this work, we extend the cytological evidence cited above to the molecular level and demonstrate that ecdysone can indeed induce amplification. Additionally, we report the discovery of a putative ecdysone response element (EcRE), which can be bound by the *Sciara* ecdysone receptor, in the 1-kb origin near the origin recognition complex (ORC)-binding site (Bielinsky et al., 2001).

Materials and methods

Larvae

S. coprophila larvae were reared in the laboratory at 21°C. Female fourth instar larvae were used for all the experiments described in this work because they are slightly larger than males and have larger polytene chromosomes, having undergone one more endocycle. Late fourth instar larvae can be staged under a compound microscope by counting the number of pigment granules in the larval eyespots, the anlage to the adult eye. The developmental stage is expressed as the number of granules in the primary row times the number of secondary rows less one (Fig. 4A) (Gabrusewycz-Garcia, 1964; Wu et al., 1993). II/9A amplification occurs at stages 10×5 and 12×6, while transcription and morphological puffing occur at stage 14×7 (Wu et al., 1993).

Salivary gland culture

Pre-amplification stage salivary glands (7×3) were dissected into Cannon's medium (GIBCO, Carlsbad, CA) (Cannon, 1964) supplemented with 60 mg glutamine and 1.5 ml of 100× penicillin, streptomycin and fungizone stock solution (GIBCO, Carlsbad, CA) per 100 ml of medium (pH 6.4). The glands were incubated in sterile polystyrene 96-well plates at 12°C to suppress bacterial and fungal contamination. At this temperature, 7×3 glands take at least 72 h to reach amplification. Two pairs of glands (with fat body still attached) were placed in each well with 0.2 ml of medium and sealed with parafilm. 20-Hydroxyecdysone (Sigma, St. Louis, MO) stock solution (1 mg/ml in ethanol) was added to a final concentration of 5 μg/ml. Controls received an equal volume of ethanol. In inhibitor studies, actinomycin D (Sigma, St. Louis, MO) was added to 40 nM (low concentration) or 900 nM (high concentration) and cycloheximide to 70 μM. The culture experiments reported here used Cannon's medium that was 10 years old and replicate experiments with that medium were reproducible. However, transcription was not induced when a new batch of medium was used unless it was aged for 16 days at room temperature, though still not reaching the level observed previously (data not shown). This suggests that some factor in the new medium changed over time that promoted transcription.

Generally, 10–15 pairs of salivary glands were used for each lane of a Southern blot. Genomic DNA isolation and quantitative genomic Southern blot hybridization were carried out as described previously (Wu et al., 1993). Total RNA from salivary glands was isolated with an RNA extraction kit (Pharmacia, Piscataway, NJ) following the manufacturer's instructions. Two micrograms total RNA, as measured by spectrophotometry, was used as template for primer extension as described previously (DiBartolomeis and Gerbi, 1989). Primer B was used for all reactions. All experiments were repeated two or more times.

Ecdysone injection

Thirty-two nanoliters 20-hydroxyecdysone in 50% ethanol (containing 25 mg/ml blue dextran as a tracer) was microinjected into the hemolymph in the posterior third of late fourth instar larvae. The amount of hormone injected was varied by changing the concentration of the solution injected. Controls were injected with an equal volume of 50% ethanol (with blue dextran tracer). Larvae were incubated at 21°C in small Petri dishes on 2.2% Bacto agar (Becton Dickinson Company, Sparks, MD). Salivary glands from individual larvae were dissected in Robert's CR buffer (Robert, 1971).

Real-time PCR

Genomic DNA was prepared with the Puregene Genomic DNA Purification Kit (Gentra, Minneapolis, MN) following the manufacturer's instructions. As controls, genomic DNA was also isolated from about 10 pre- and post-amplification stage salivary glands and from 10 to 20 adult female flies. Real-time PCR was performed using the following primers to detect the II/9A 1-kb ORI (oII/9A RT for set 6: 5′-TGTAATATATGCAACACGAGGCG-3′; oII/9A RT rev set 6: 5′-TTCGGCTTAGAACGATGCACT-3′) and RNA puff III/9 (oSD159 RT for set 2: 5′-GGATAGCTTCTTGTTCACACCCTC-3′; oSD159 RT rev set 2: 5′-TCGTAGGCGTTTTTCGCTTC-3′). Fold amplification was calculated by comparing the ratio of the relative abundance of II/9A:III/9 in injected larvae to the ratio of II/9A:III/9 in adult flies where there is no amplification and the ratio of the two loci is set to one.

In experiments where both amplification and transcription were assayed, the salivary glands were homogenized in the Gentra kit cell lysis buffer. One half of the sample was RNase treated and used to prepare genomic DNA. The other half was prepared without RNase treatment and the genomic DNA was removed by degradation with Amplification Grade DNase (Invitrogen, Carlsbad, CA). Reverse transcription of the total RNA was performed using a TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and the resulting cDNA used for real-time PCR reactions with the following primer sets to detect gene II/9-1 (ogene I RT for set 1: 5′-GCCGAAATCAAACGACTCGA-3′; ogene I RT rev set 1: 5′-TAGAGCGTTTTCGCAGGCA-3′) and 18S rRNA (oSc18 RT for set 2: 5′-AAAGGAATTGACGGAAGGGC-3′; oSc18 RT rev set 2: 5′-AAATTAAGCCGCAGGCTCC-3′). Real-time PCR was performed on an ABI 7300 Real-time PCR System (Applied Biosystems, Foster City, CA). Each reaction was performed in triplicate.

Electromobility shift assays

About 100–200 pairs of puff stage salivary glands were dissected and stored on ice in Robert's CR buffer (Robert, 1971). *Sciara* salivary gland nuclear extracts were prepared as described (Schreiber et al., 1989). *Sciara* EcR-A, EcR-B and USP cDNAs (J. Johnson et al., personal communication) and *Drosophila* EcR-A and USP were transcribed and translated in vitro using the TnT T7 Quick Coupled Transcription/Translation System (Promega; Madison, WI). Expression vectors for the *Drosophila* proteins (pCMX-EcR and pCMX-USP) were a gift from Ronald Evans. All gel shift reactions were carried out in 25 μl of binding buffer (22.5 mM HEPES pH 7.0, 1 mM Tris pH 7.5, 90 mM KCl, 0.2 mM EDTA, 9% glycerol, 1 mM DTT and either 0.5 mM PMSF or 1× Complete Mini Protease Inhibitor Cocktail [Roche; Mannheim, Germany]). In some cases, polyclonal antibodies to *Drosophila* EcR or DHR3 (gift from Lucy Cherbas) or monoclonal antibodies to *Drosophila* EcR-A (15G1a) and EcR-B1 (AD4.4) (gift from David Hogness) (Talbot et al., 1993) were added to induce a supershift. The following probe sequences were used: gene II/9-1 EcRE1 (5′-TCGAAGCTGTATTTCCGTTGAACAAATTAAA-3′), gene II/9-1 EcRE2 (5′-TCGAAAAGGTGGACATATTGACCAAAAATTG-3′), gene II/9-1 EcRE3 (5′-TCGAAGAAACGTACGAACTGACCTTCTAACC-3′), DmHsp27 EcRE (5′-CGAAGCGAGACAAGGGTTCAATGCACTTGTC-3′) and ORI EcRE (5′-TTGCTTCAGTAGCACACATTGACCTCTATAA-3′). Probes were ^{32}P-CTP labeled with the large fragment (Klenow) of DNA polymerase I (New England Biolabs, Beverley, MA). In Fig. 6A, the nonspecific competitor had the sequence: 5′-TCGACGCGGCCGCTTCAGTTAGCGGTAC-3′. In Figs. 6D and E, the triangle represents 25-, 50-, 100-, 150- and 200-fold excess unlabeled competitor.

Results

Ecdysone induces transcription in cultured salivary glands

The promoter of gene II/9-1 is known to be ecdysone responsive in transgenic *Drosophila* salivary glands (Bienz-Tadmor et al., 1991). To determine if ecdysone can induce transcription in *Sciara*, salivary glands from pre-amplification stage larvae (7×3) were explanted into Cannon's medium (Cannon, 1964), known to support DNA puffing in *Sciara* (Cannon, 1965), either in the presence (5 μg/ml) or absence of 20-hydroxyecdysone (hereafter referred to as ecdysone). Total RNA harvested after the times indicated was used as template for primer extension (Fig. 2). 14×7 stage salivary glands, the stage when transcription normally occurs, served as a positive control. The primer used detected both gene II/9-1 and II/9-2 (DiBartolomeis and Gerbi, 1989). Although both genes are ecdysone responsive, gene II/9-1 is the predominant transcript, as evident from the intensity of the bands in Fig. 2A. Ecdysone-induced transcription was observed as early as 3 h (Fig. 2B) and was induced robustly after 4 h, continuing strongly up to 48 h (Fig. 2A). In contrast, no transcription was observed until 48 h in the absence of hormone (Fig. 2A). When the glands were incubated with cycloheximide, transcription was negligible (Fig. 2B), suggesting that protein synthesis is required to induce robust transcription. This is consistent with previous work showing that cycloheximide inhibited ecdysone-induced transcription from the gene II/9-1 promoter in transgenic *Drosophila* (Bienz-Tadmor et al., 1991).

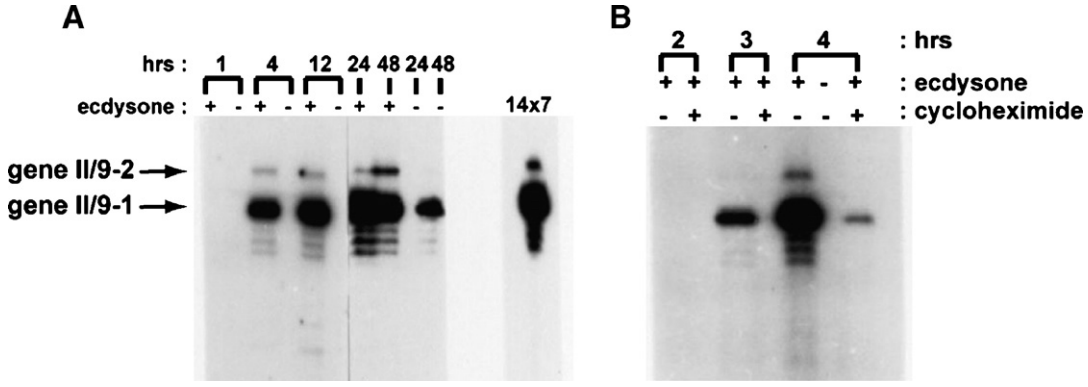


Fig. 2. Ecdysone induces transcription in cultured salivary glands. (A, B) Primer extension on total RNA from pre-amplification stage (7×3) salivary glands cultured at 12°C under various conditions. The bands for genes II/9-1 and 9-2 are indicated. As a control, primer extension was performed on total RNA from uncultured 14×7 salivary glands.

Ecdysone induces amplification in cultured salivary glands

To determine if ecdysone induces amplification in culture, 10–15 pairs of salivary glands from pre-amplification (7×3) larvae were cultured in the presence (5 μg/ml) or absence of ecdysone. Amplification was assayed by Southern blots using probes specific to the II/9A locus and an RNA puff at locus III/9 that does not amplify (Wu et al., 1993), which served as a loading control. The autoradiographs were analyzed by densitometry and the ratio of the bands (II/9A: III/9) was calculated. Amplification was determined by comparing the ratio for ecdysone-treated samples to the untreated control. Slight amplification was observed after 1 day (~2-fold) and substantial amplification (~12-fold) after 2 days (Fig. 3A). When earlier pre-eyespot stage glands were tested, however, ecdysone failed to induce amplification (Fig. 3C), suggesting that there is a developmental threshold which must be surpassed before amplification can occur. Additionally, ecdysone was unable to induce further amplification in glands from post-amplification stage larvae (eyespot stage 14×7; Fig. 3D). When incubated with a concentration of the transcription inhibitor actinomycin D (AMD) that only inhibits rRNA transcription, ecdysone still induced some amplification (Fig. 3B). However, at a higher concentration of AMD, which blocks both rRNA and mRNA transcription, ecdysone was unable to induce amplification. Moreover, when treated with the protein synthesis inhibitor cycloheximide, ecdysone no longer induced amplification (Fig. 3B). These data suggest that some factor must be produced de novo for amplification to occur after exposure to ecdysone.

Ecdysone induces amplification in vivo

In order to see if ecdysone could prematurely induce amplification in a more physiological setting, 32 nl of 1 mg/ml ecdysone (32 ng/larva) in 50% ethanol was injected into the hemolymph of individual pre-amplification stage larva.

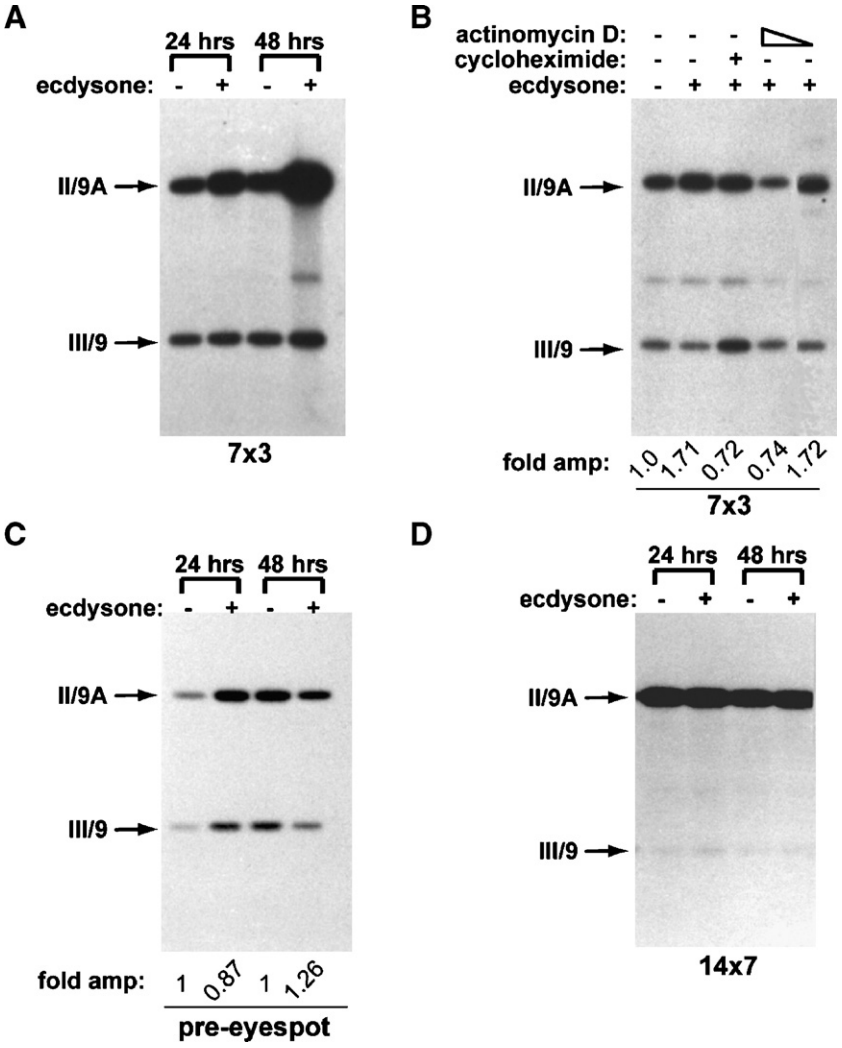


Fig. 3. Ecdysone induces amplification in cultured salivary glands. (A–D) Salivary glands from 7×3 (A and B), pre-eyespot (C) and 14×7 (D) stage larvae were explanted into Cannon's medium and incubated for the indicated times and treatments at 12°C. Southern blots of EcoRI digested salivary gland genomic DNA were prepared and probed with sequences specific to DNA puff gene II/9-1 and the III/9 RNA puff (unamplified control). The extra band in panels A and B is the result of cross-hybridization of the gene II/9-1 probe to the fragment containing gene II/9-2 (Wu et al., 1993) which is 85% similar to gene II/9-1 (DiBartolomeis and Gerbi, 1989).

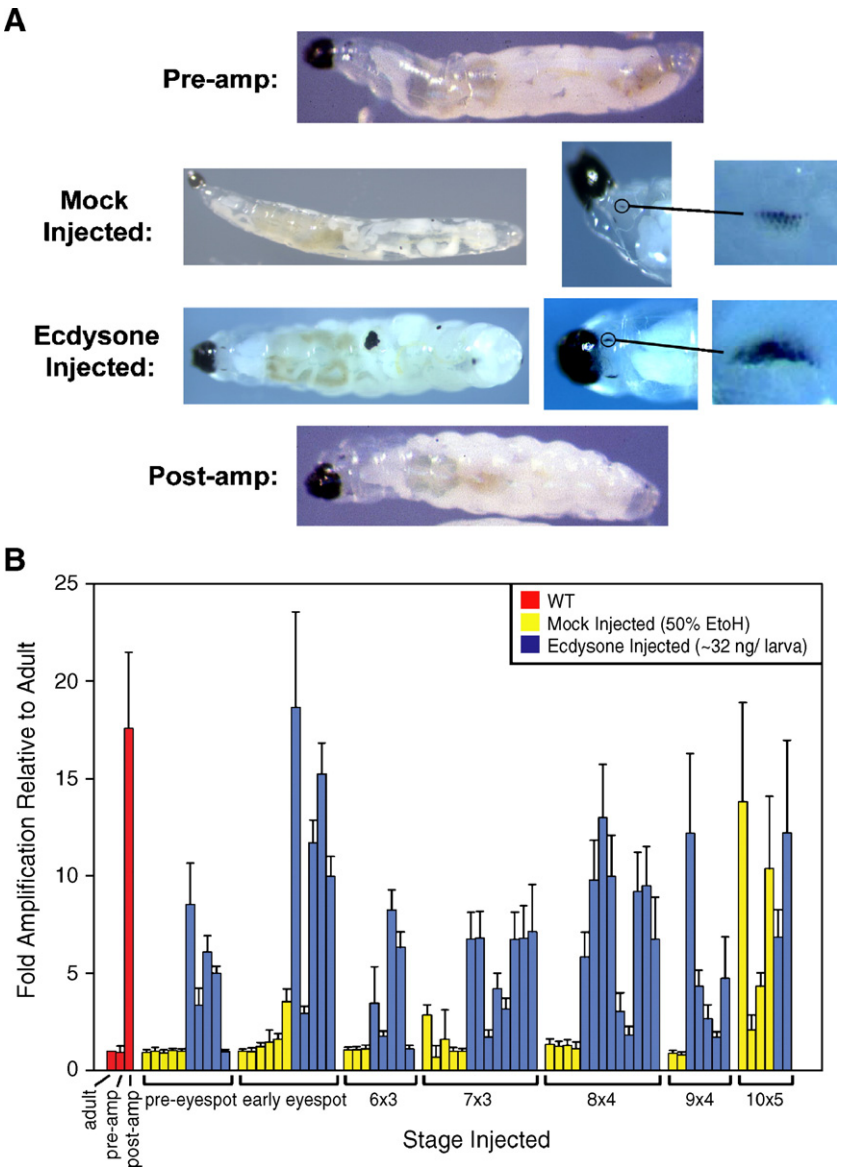


Fig. 4. Ecdysone induces amplification in vivo. Larvae of the stage indicated were either mock injected or injected with 32 ng 20-hydroxyecdysone into the hemolymph and incubated 24 h at 21°C. (A) Micrographs of larvae showing the phenotype of uninjected pre- and post-amplification larva compared to mock and ecdysone-injected 8×4 larvae after 24 h. The insets on the right are enlargements of the larval eyespots. (B) Salivary glands were harvested from each individual injected larva and the DNA was prepared and used as template for real-time PCR using primers specific to the II/9A locus and the III/9 locus (unamplified control). Salivary gland DNA from 10 larvae was pooled for the wild-type controls. Data are expressed as fold amplification relative to adult DNA and each bar represents an individual injected larva. All samples were run in triplicate and the error bars represent real-time PCR error.

Matched mock-injected controls were injected with 50% ethanol. The larvae were incubated at 21°C for 24 h. Ecdysone-injected larvae prematurely took on the morphology of post-amplification late larval/pre-pupal stages (Fig. 4A). The larvae wandered less and their bodies became more opaque. Significantly, their eyespot granules began to prematurely coalesce and migrate laterally, reminiscent of a post-amplification stage called “edge-eye” (see insets in Fig. 4A). Mock-injected control larvae developed normally. They remained highly mobile, their bodies remained transparent and their eyespot granules remained distinct and close to the dorsal midline.

After 24 h, the salivary glands from each injected larva were harvested. Genomic DNA was prepared and used as template for real-time PCR with primers specific to the DNA puff II/9A 1-kb ORI and the III/9 RNA puff locus. The extent of amplification was determined by comparing the ratio of signal for the two loci with that found for adult DNA where there is no amplification (the ratio between the two loci is one). Pooled DNA from ten pre-amplification or ten post-amplification stage salivary glands were used as controls. Pre-amplification larvae exhibited no amplification at the II/9A locus while post-amplification larvae amplified ~17-fold, corresponding with the level observed previously by quantitative Southern blots (Wu et al., 1993). At all stages, amplification could be observed in ecdysone-injected larvae after 24 h (Fig. 4B). However, amplification was not observed in every individual likely due to several factors: developmental heterogeneity, accuracy of injection and amount

of material lost from the injection site. The level of amplification rarely approached the natural level (~17-fold). Importantly, robust amplification was never observed in mock-injected larvae, with the notable exception of larvae injected at eyespot stage 10×5. Since these larvae are about to initiate amplification anyway, this observation was expected and shows that the ethanol in the injection had no effect on their natural progression. Taken together, these results demonstrate that ecdysone can prematurely induce amplification in vivo.

Ecdysone-induced amplification precedes transcription in vivo

DNA amplification precedes transcription at the II/9A locus during normal development (Wu et al., 1993). However, the normal timing was reversed in the cultured salivary gland experiments. Was this also the case in ecdysone-injected larvae? In the experiment above (Fig. 4B), 32 ng ecdysone per larva, an amount known to induce DNA puffing (Crouse, 1968), was sufficient to induce amplification. Assuming a total larval volume of about 2.5 µl, injection of this amount corresponds to a concentration of approximately 13 µg/ml. A concentration of 5 µg/ml was used in the culture experiments. It was possible that this concentration immediately activated transcription prior to amplification, leading to the aberrant timing. Hence, the threshold concentration of ecdysone that would stimulate amplification was determined. A dilution series revealed that 320 pg/larva (~130 ng/ml) was sufficient to induce robust amplification while a 10-fold further dilution (32 pg/larva or ~13 ng/ml) only produced low levels of amplification (Fig. 5A and data not shown). This concentration corresponds well with

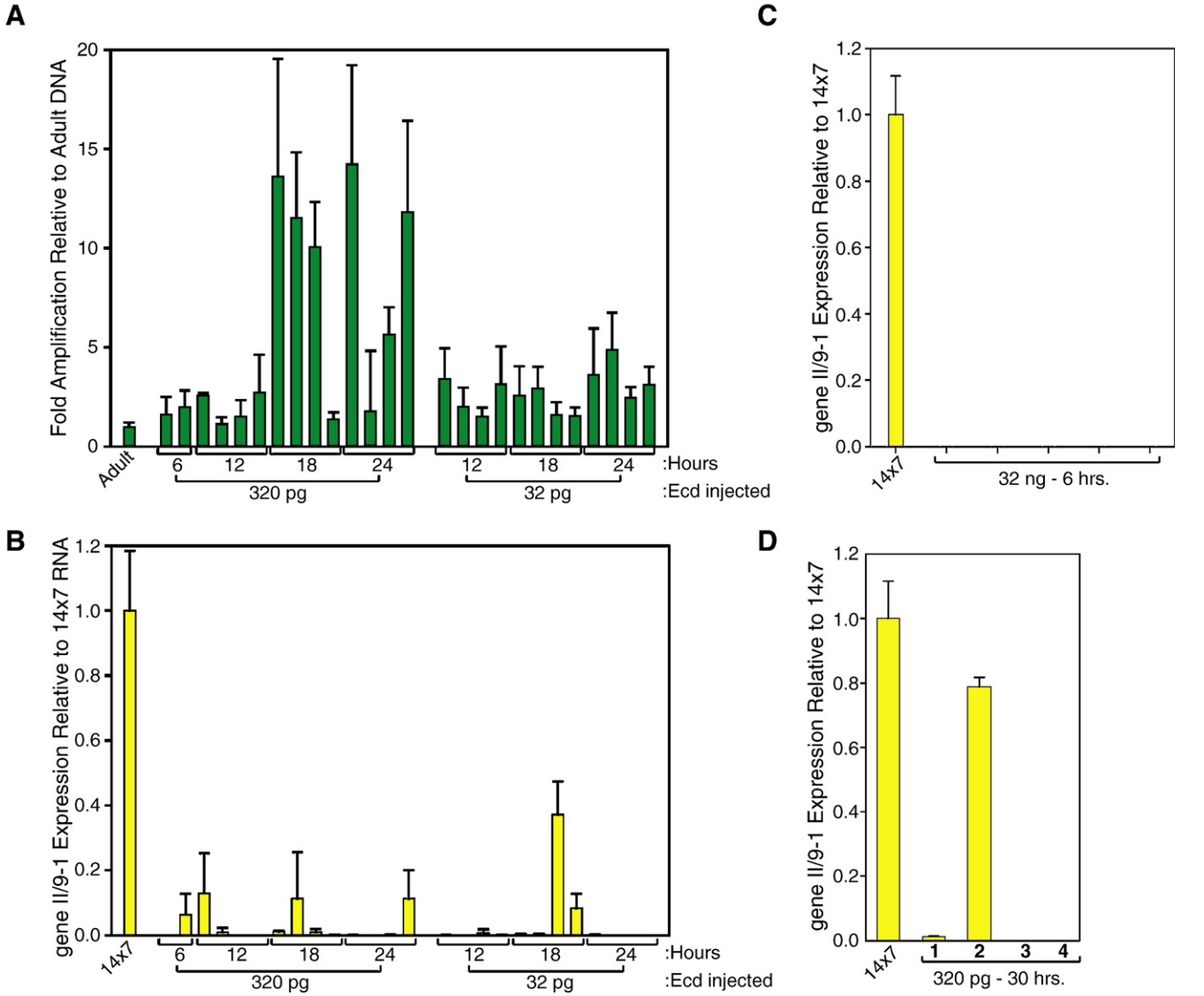


Fig. 5. Ecdysone-induced amplification precedes transcription in vivo. (A, B) Larvae were injected with the indicated amount of ecdysone and the salivary glands harvested after incubation at 21°C after the time indicated. Both DNA and total RNA were prepared from each individual larvae and assayed by real-time PCR. (A) Genomic DNA was used as template to assess the extent of amplification as described in Fig. 4. (B) Total RNA was reverse transcribed and used as template for real-time PCR using primers specific to genes II/9-1 and II/9-2 and *Sciara* 18S rRNA (control). The results are expressed relative to the RNA content of 14×7 stage salivary glands (set to one). All samples were run in triplicate and the error bars represent real-time PCR error.

that (~50–250 ng/ml) necessary to induce RNA puffing in *Drosophila* (Ashburner et al., 1974). Using this threshold concentration (320 pg/larva), a time course assaying for both amplification and transcription was performed (Fig. 5). Larvae were injected as described above and incubated at 21°C for the times indicated. Their salivary glands were harvested and both DNA and total RNA prepared. The DNA was used to determine amplification as described above. Amplification was first observed 18 h post-injection (320 pg/larva) (Fig. 5A). Total RNA was prepared for real-time PCR analysis using primers specific to gene II/9-1 and 18S rRNA as a control. The results are expressed relative to the RNA content of 14×7 eyespot stage salivary glands (Wu et al., 1993). Robust transcription of gene II/9-1 was not seen until 30 h post-injection (Figs. 5B, D)

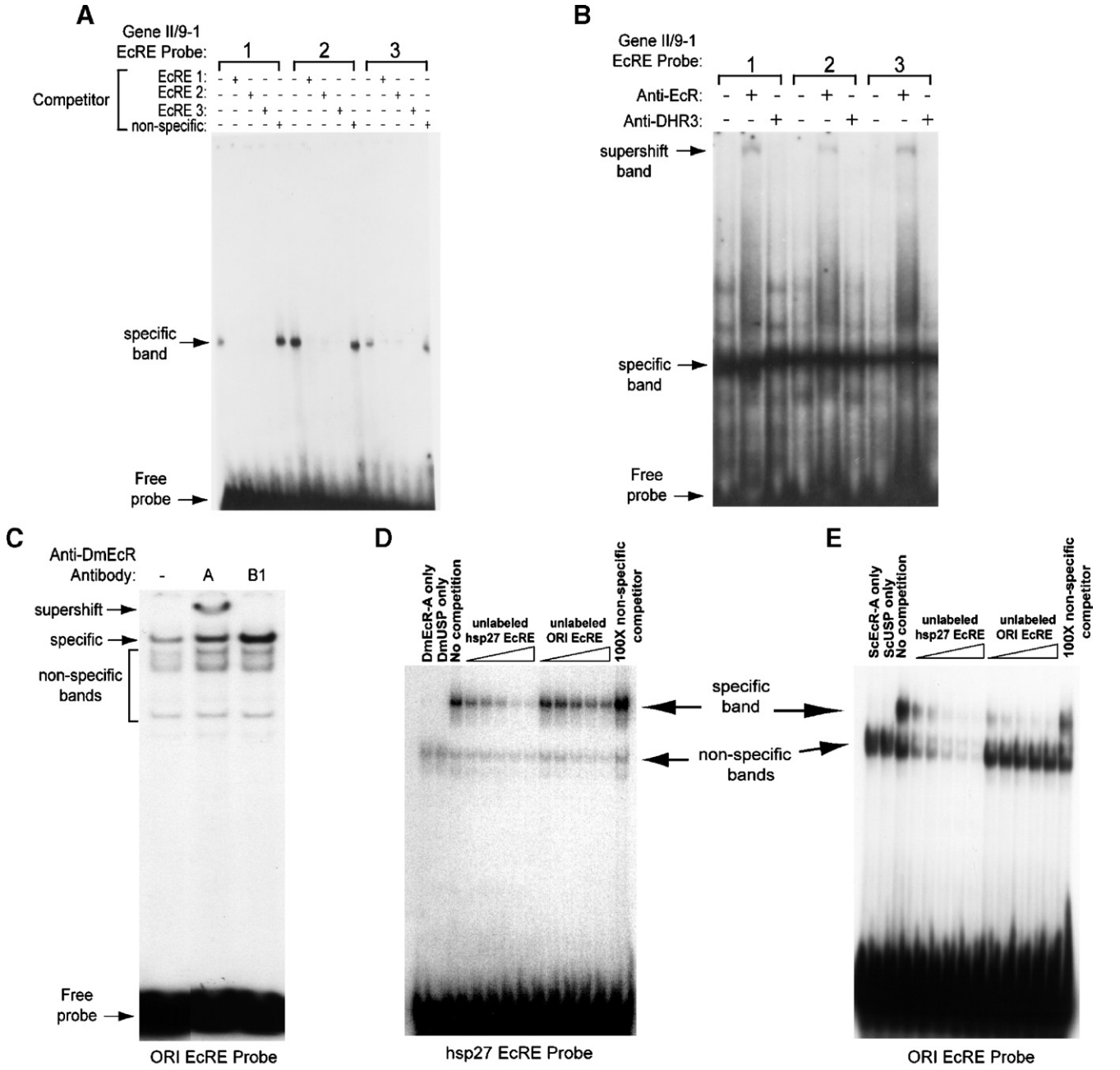


Fig. 6. The *Sciara* ecdysone receptor binds *Sciara* EcREs. (A, B) *Sciara* salivary gland nuclear extracts were prepared and incubated with radiolabeled probes carrying the gene II/9-1 promoter EcREs (see Materials and methods) in electromobility shift assays (EMSA). The position of the free probe and specifically shifted bands are indicated. (A) The EcRE being shifted in each lane is indicated at the top and the competitor used is indicated at the side. (B) Same as in panel A except that anti-DmEcR or anti-DmDHR3 antibodies were included to induce a supershift (indicated by arrow). (C) *Sciara* salivary gland nuclear extract was prepared and used in an EMSA against the radiolabeled ORI EcRE. Both specific and nonspecific bands are indicated. Anti-DmEcR-A and anti-DmEcR-B1 antibodies included to induce a supershift are indicated at the top. (D) *Drosophila* EcR-A and USP proteins were in vitro transcribed and translated and used in an EMSA against radiolabeled hsp27 EcRE. (E) *Sciara* EcR-A and USP proteins were in vitro transcribed and translated and used for EMSA against radiolabeled ORI EcRE. In both panels D and E, unlabeled hsp27 and ORI EcREs were used as competitors at increasing concentrations. The position of free probe, bands specifically shifted by EcR/USP and bands shifted nonspecifically by the transcription/translation mixture are indicated.

and that was in only one of four larvae (2 in Fig. 5D). Therefore, transcription does not occur until at least 30 h after ecdysone injection, considerably past the time when DNA amplification is induced (Fig. 5A). Importantly, injection of 32 ng ecdysone failed to induce any significant transcription at 6 h (Fig. 5C), a time when strong transcription was observed in culture with a similar concentration of ecdysone. Transcription of the II/9A genes naturally occurs in a short window during the 14×7 eyespot stage and little transcript remains by the time the larvae reach the edge eye stage (Wu et al., 1993). Two of the 30-h larvae exhibited the edge eye phenotype when their glands were harvested (3 and 4 in Fig. 5D), while the other two still had distinct eyespots (1 and 2 in Fig. 5D) suggesting that transcription only occurs in a brief window in the injected larvae, as well. These results indicate that amplification and transcription timing occur normally in injected larvae.

The II/9A 1-kb origin contains a putative EcRE

DNA sequence analysis of the II/9A locus, performed in light of the above results, revealed several putative ecdysone response elements (EcREs; Fig. 1B). Importantly, in addition to the EcREs anticipated to be in the promoters of genes II/9-1 and II/9-2, an EcRE in the 1-kb ORI just upstream of the ORC-binding site was also identified (Fig. 1A). EcREs are highly degenerate, pseudo-palindromic ecdysone receptor-binding sites. The *Drosophila* ecdysone receptor will bind EcREs with more than one nucleotide between the half sites, but clearly prefers EcREs with a single nucleotide separating the half sites (Antoniewski et al., 1993). However, all the putative *Sciara* EcREs had more than one nucleotide separating their half sites raising the question of whether they could perform such a role in *Sciara*.

ScEcR binds ScEcREs

To determine if these EcREs were bona fide *Sciara* EcREs, electromobility shift assays (EMSA) were performed. Radiolabeled probes containing one of the three EcREs identified in the promoter of gene II/9-1 were shifted when incubated with puff stage *Sciara* salivary gland nuclear extract (Fig. 6A). The specificity of the shift is indicated by the fact that unlabeled probes compete specifically (e.g., unlabeled EcRE1 competes specifically for binding to labeled EcRE1). Each of the EcREs competes well for binding to the others (e.g., EcRE2 competes for binding to EcRE1 and vice versa). Addition of an antibody specific to DmEcR to the reaction caused a supershift (Fig. 6B). In contrast, antibodies to DmDHR3, a close homolog of DmEcR (Koelle et al., 1991), failed to produce a supershift, suggesting that the component in the nuclear extract responsible for the shift was the ecdysone receptor.

A radiolabeled probe containing the ORI EcRE was shifted when incubated with puff stage salivary gland nuclear extract (Fig. 6C) and a monoclonal DmEcR-A isoform-specific antibody supershifted this band. Conversely, antibodies specific to DmEcR-B1 failed to supershift the ORI EcRE, suggesting that the shift is specific for the A isoform. Furthermore, both the A and B isoforms of *Sciara* EcR (ScEcR-A and ScEcR-B) and ultraspiracle (ScUSP; J. Johnson et al., personal communication) were cloned. We also obtained clones for the *Drosophila* EcR-A and USP (DmEcR-A and DmUSP) proteins (Koelle et al., 1991; Talbot et al., 1993; Yao et al., 1992). The proteins were transcribed and translated in vitro and used in reciprocal EMSAs against both *Drosophila* hsp27 EcRE (Riddihough and Pelham, 1987) and ORI EcRE probes. DmEcR-A and DmUSP shift the hsp27 probe only when both are present (Fig. 6D). Unlabeled hsp27 EcRE can compete for this binding while unlabeled nonspecific competitor (pBluescript SK-) has no effect, indicating that the competition is specific. However, the *Sciara* ORI EcRE competed poorly for binding, suggesting that DmEcR prefers to bind the hsp27 EcRE, consistent with the literature that the *Drosophila* receptor prefers a single nucleotide between the half sites (Antoniewski et al., 1993). In the converse experiment, ScEcR-A and ScUSP only shifted the ORI EcRE when both were present (Fig. 6E). The specificity of this shift was demonstrated by the ability of unlabeled ORI EcRE to compete for binding. Importantly, unlabeled hsp27 EcRE competed equally well, demonstrating that ScEcR has an equal affinity for both EcREs. These results suggest that ScEcR has a relaxed requirement for the number of nucleotides separating the half sites. Hence, the ORI EcRE could be an authentic *Sciara* EcRE.

Discussion

Initiation of DNA replication is tightly controlled by the cell and each replication origin fires once and only once to ensure the precise duplication of the genome. However, locus-specific DNA amplification occurs in the salivary gland polytene chromosomes of the lower dipteran fly, *S. coprophila*, during the normal course of development. How this regulation is overridden to permit repeated rounds of origin re-firing is unknown. In this report, we demonstrate that ecdysone induces DNA amplification at *Sciara* DNA puff II/9A. It has been shown previously that ecdysone induces transcription (Bienz-Tadmor et al., 1991) and the concomitant decondensation of chromatin to form DNA puffs in sciarid salivary gland polytene chromosomes (Amabis and Amabis, 1984a; Crouse, 1968; Stocker and Pavan, 1974). We demonstrate here that injection of ecdysone leads to premature amplification and transcription at the II/9A locus. Amplification precedes transcription in injected larvae, mirroring the normal developmental sequence. Ecdysone also induces premature DNA amplification in cultured salivary glands within a narrow developmental window, with post-amplification stage salivary glands incubated with ecdysone failing to amplify further. However, the normal temporal profile of amplification and transcription was not maintained in the culture experiments. One could propose several reasons for this discrepancy. First, incubating the glands at a lower temperature (12°C) may delay the onset of amplification or promote early transcription in some manner. Another possible explanation lies in the concentration of ecdysone used in these experiments. In *Drosophila*, different puffs appear to respond to different levels of ecdysone (Karim and Thummel, 1992).

Perhaps II/9A amplification and transcription respond to different levels. We incubated the salivary glands with 5 μg/ml ecdysone. However, in *Drosophila* puffing is induced at ~50–250 ng/ml (Ashburner et al., 1974). Significantly, we found that a concentration of ~130 ng/ml was sufficient to induce amplification and transcription in whole larvae with the correct timing. The high ecdysone concentration used in culture may have induced transcription before amplification could get started. On the other hand, injection of a high concentration (~13 μg/ml) into larvae did not induce transcription prematurely. This result suggests that the aberrant timing was not due to ecdysone concentration, but simply an artifact of culturing the glands because normal timing was observed in vivo.

What accounts for the normal developmental timing? We know that RNA polymerase is loaded in an inactive form at the promoter for gene II/9-1 during amplification (Lunyak et al., 2002), suggesting that a transcription complex is present at the promoter at this time. Therefore, it appears that ecdysone promotes the initiation of amplification and the recruitment of polymerase to the promoter of gene II/9-1 concurrently, but transcription is prevented during active DNA synthesis either by the presence of a repressor or the absence of an activator. Subsequently, an ecdysone-induced factor could remove the repressor or provide the activator (or both) in a temporally specific manner, an idea we have proposed before (Gerbi et al., 1993). Further investigation is necessary to unravel these possibilities.

Is Sciara DNA puff II/9A an early or late puff?

In *Drosophila*, Ashburner and colleagues (1974) proposed a model in which ecdysone immediately induces early puffs that produce transcription factors which trigger late puffs. Hence, late puffs were defined as requiring protein synthesis for their induction. *Sciara* DNA puffs have some features of both early and late puffs as defined by Ashburner. The presence of putative EcREs in the II/9A 1-kb ORI as well as the promoters of the II/9A genes, which bind the *Sciara* ecdysone receptor, fits the definition of an early puff. On the other hand, we previously demonstrated that the promoter for gene II/9-1 acts like a late puff in transgenic *Drosophila* larvae because it requires protein synthesis after ecdysone exposure to become active (Bienz-Tadmor et al., 1991). In this study, we confirmed this observation in *Sciara* salivary glands. Moreover, DNA amplification in *Sciara* appears to be similarly regulated. In addition, cycloheximide blocked amplification when injected with ecdysone into another sciarid fly, *Trichosia pubescens*, though the results of these experiments were difficult to interpret because cycloheximide was highly toxic to the larvae (Amabis and Amabis, 1984a). Evidence from *Drosophila* suggests that ecdysone up-regulates expression of the ecdysone receptor (Karim and Thummel, 1992; Koelle et al., 1991). Therefore, it may be necessary to first express the ecdysone receptor, or a cofactor, to sufficient levels to induce amplification and transcription. In support of this possibility, the level of *Sciara* EcR-A increases in salivary glands at amplification stage (J. Johnson et al., personal communication).

Is the ecdysone receptor the amplification factor in Sciara?

DNA amplification occurs when cyclin E levels are high, a condition repressive to pre-RC formation (Calvi et al., 1998). The fact that re-replication leading to amplification happens at just a few specific loci and not genome-wide suggests that an amplification factor may be tethered at these loci. The data presented here suggest that the ecdysone receptor could be the elusive amplification factor in developmentally regulated *Sciara* DNA puff amplification. This would be the first example of a hormone receptor regulating DNA replication. In particular, the presence of an EcRE adjacent to the ORC-binding site in the II/9A 1-kb ORI suggests a direct effect of ecdysone on amplification. This EcRE has more than one nucleotide separating the two half sites. Results from electromobility shift assays revealed that this EcRE is bound by the *Sciara* EcR, but is not a preferred substrate for the *Drosophila* EcR. It would appear that the *Sciara* EcR has a relaxed specificity for the number of nucleotides separating the two half sites of an EcRE relative to *Drosophila*. However, the results with transcription and protein synthesis inhibitors point to an indirect mechanism for the action of ecdysone in amplification. Interestingly, the level of EcR rises in *Sciara* salivary glands at amplification stage (J. Johnson et al., personal communication). Therefore, the effect of ecdysone on amplification could be both direct and indirect, requiring first the ecdysone-induced production of the EcR at amplification stage followed by binding of the EcR to the ORI EcRE where it directs the initiation of amplification. However, in vivo evidence to validate this theory must await development of a transformation system in *Sciara* and production of *Sciara*-specific EcR antibodies. The fact that a *Drosophila* anti-EcR-A antibody, and not anti-EcR-B, produced a supershift in an EMSA with salivary gland nuclear extract suggests the amplification factor would be specifically EcR-A. In addition, we have preliminary data that strongly suggests EcR-A is the predominant isoform in *Sciara* salivary glands (J. Johnson et al., personal communication). In contrast, EcR-B is the predominant isoform in *Drosophila* larval tissues including salivary glands (Talbot et al., 1993), although it has been suggested recently that the salivary glands may contain a small amounts of EcR-A (Davis et al., 2005).

A growing body of evidence has begun to unravel the roles that transcription factors play in the regulation of DNA replication in eukaryotes. Kohzaki and Murakami (2005) have written that the term transcription factor may be a misnomer and that these proteins are better thought of as "general regulators of the formation of functional complexes on specific chromatin sites." Many of the replication origins in budding yeast (autonomously replicating sequences; ARSs) contain binding sites for transcription factors which can function to either promote or repress replication (Hu et al., 1999; Kohzaki et al., 1999; Li et al., 1998; Marahrens and Stillman, 1992). For example, the B3 element of ARS1 is a binding site for the transcription factor Abf1 and is required for efficient initiation (Marahrens and Stillman, 1992). Recently, an important finding

demonstrated that addition of a transcription factor-binding site to an episome focused replication initiation events near that binding site when the transcription factor was present, even in the absence of transcription (Danis et al., 2004). Similarly, we observed that the initiation zone at the II/9A locus contracts to the 1-kb ORI during amplification, coinciding with loading of the transcription machinery at the promoter for gene II/9-1 (Lunyak et al., 2002).

Transcription factors have been implicated in amplification at the chorion loci in *Drosophila* as well. For example, Myb and its binding partners Mip120, Mip130 and Mip30 along with the chromatin assembly factor 1 (Caf1) subunit p55 form a complex that binds to ACE3 and oriβ where it interacts with ORC (Beall et al., 2002). ACE3 contains both Myb and Mip120-binding sites which when deleted prevent amplification. Interestingly, in Mip130 mutants instead of amplification the entire genome is replicated (Beall et al., 2004). These data taken together suggest the Myb/Mip/Caf1 p55 complex acts to repress genomic replication with Myb acting as a switch to turn on replication specifically at the chorion loci.

The S phase transcription factor complex of E2F/DP1/Rbf1 also plays a role in chorion locus amplification. *Drosophila* has two isoforms of E2F. E2F1 colocalizes with ORC at ACE3 and E2F1 null mutants fail to amplify (Bosco et al., 2001; Royzman et al., 1999). Mutants of the retinoblastoma protein (Rbf1) exhibit increased amplification suggesting that Rbf1 and E2F1 form a complex that represses amplification (Bosco et al., 2001), a conclusion reinforced by the finding that E2F1 mutants that cannot bind Rbf1 also overamplify (Royzman et al., 1999). Mutants of E2F2, Rbf1 and DP1 all exhibit genomic replication instead of amplification, suggesting that the E2F2 complex plays a role in repressing genomic replication outside of the chorion loci (Cayirlioglu et al., 2001). Interestingly, mutants in these same proteins also derepress expression of pre-replication complex proteins which might account for the ectopic DNA replication (Cayirlioglu et al., 2003). Both Myb and the E2F transcription factors are targets of the cell cycle machinery, promoting the initiation of amplification through some currently unknown signal.

How might the ecdysone receptor regulate amplification?

The identification of an EcRE immediately adjacent to the binding site for ORC raises the intriguing possibility that the receptor could directly recruit ORC or stabilize its interaction with the 1-kb ORI. Viral transcription factors are known to actively recruit initiation factors to viral origins. For example, the polyomavirus origin contains a binding site for c-Jun, a component of the AP1 transcription factor, which directly recruits the viral initiator protein complex, large T antigen (Ito et al., 1996; Murakami et al., 1991). Furthermore, the Epstein–Barr virus recruits cellular ORC to its replication origin through a direct interaction with the transcription factor EBNA1 (Chaudhuri et al., 2001; Dhar et al., 2001; Ritzi et al., 2003; Schepers et al., 2001).

On the other hand, transcription factors also recruit proteins which influence chromatin structure. For example, a repressive chromatin environment blocks SV40 replication and transcription factors can reverse this inhibition (Cheng and Kelly, 1989; Cheng et al., 1992). In *Drosophila* follicle cells, histones in the chorion loci become acetylated at the same time ORC2 binds (Aggarwal and Calvi, 2004). This acetylation did not coincide with BrdU incorporation at the replication forks, arguing it reflected acetylation after histone assembly. In flies mutant for the histone deacetylase, Rpd3, ectopic genome wide acetylation and replication occurred at a time when amplification should occur. Moreover, tethering Rpd3 near the origin inhibited amplification, suggesting that amplification is indeed sensitive to chromatin modification (Aggarwal and Calvi, 2004).

The ecdysone receptor is known to bind to a corepressor, SMRTER, which in turn recruits histone deacetylases (Tsai et al., 1999). Intriguingly, Myb has been shown to interact with SMRT, the vertebrate homolog of SMRTER, in addition to the corepressor N-CoR (Li and McDonnell, 2002). Phosphorylation by the cell cycle machinery disrupts this interaction, allowing transcription. Perhaps a similar mechanism is employed in *Sciara* DNA amplification, comparable to *Drosophila* transcription where the interaction between the ecdysone receptor and SMRTER is disrupted by ecdysone (Tsai et al., 1999), potentially opening up chromatin structure. Although no histone acetyltransferases have been described which bind to the EcR, recently it has been reported that EcR associates with a histone methyltransferase called CARMER, which has been implicated in *Drosophila* development (Cakouros et al., 2004). Hence, the ecdysone receptor could exert its influence by (1) promoting a chromatin environment conducive to the binding of replication factors and/or (2) directly recruiting ORC to the 1-kb ORI. Further work is required to identify which mechanism is used or whether both contribute to regulation of amplification during larval development.

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